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# Monitoring for Cytomegalovirus Infection in Organ Transplant Recipients: Analysis of pp65 Antigen, DNA and Late mRNA in Peripheral Blood Leukocytes

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The use of sensitive and specific methods for rapid and reliable diagnosis is required due to the considerable impact of human cytomegalovirus (HCMV) in organ transplant recipients. For this purpose the demonstration of the presence of viral antigens in peripheral blood leukocytes (PMNLs) and of viral nucleic acids in the same cells or in sera would seem to be of valid support. The present study was designed to test pp65 antigen, HCMV DNA and HCMV late mRNA in order to provide clinical information for the management of the infection. Fifty solid organ recipients were monitored for six months after transplant. The data obtained from the various tests were analysed from the first evidence of HCMV infection revealed by positive antigenaemia and/or DNA-polymerase chain reaction (PCR). In 3 asymptomatic and in 7 symptomatic patients, PCR became positive 1–2 weeks before antigenaemia but PCR did not discriminate the clinical evolution of HCMV infection. The antigenaemia test well correlated to the development of viral infection being positive in all symptomatics and in 31, 2% of asymptomatics. The antigenic load >100/2 × $10^5$ positive cells was always associated with clinical signs of illness. The detection of late mRNA was more indicative of the virus replicative status in the follow-up of patients treated with ganciclovir. In some cases there was evidence, prior to the other two tests, the block of viral replication due to the antiviral therapy and in others the onset of HCMV infection relapse. ***J. Med. Virol. 53:189–195, 1997.***



**KEY WORDS:** HCMV, transplantation, antigenaemia, PCR, RT-PCR

## INTRODUCTION

Human cytomegalovirus (HCMV) is an ubiquitous agent that infects 60–80% of the human population. The infection in immunocompetent adults is usually asymptomatic or produces a self-limited mononucleosis syndrome [Ho, 1991] and the genome transcription is limited to the immediate early (IE) gene, so that HCMV cannot produce a viral progeny. This latent infection usually occurs in peripheral blood monocytes and in endothelial cells [Taylor-Wiedeman et al., 1991; Grefte et al., 1993; Sedmak et al., 1994; Grefte et al., 1993].

In immunocompromised conditions, either a primary infection or a reactivation of latent HCMV, can generate a productive infection which may cause the virus to spread in the host organism [Ho, 1991]. The incidence of HCMV infection among patients undergoing solid organ transplantation ranges from 23% to 85% and symptomatic disease develops in approximately half of these patients [Ho, 1993; Kanj et al., 1996]. In addition to its direct pathogenicity HCMV is considered a predisposing factor for bacterial and fungal infection because of its immunosuppressive effect. Furthermore, it has been suggested that HCMV plays a role in graft rejection, inducing class I and class II histocompatibility antigens on infected cells and increasing T-lymphocytes cytotoxic response [von Willebrand et al., 1986; Grundy et al., 1988; Pasternack et al., 1990; Rasmussen, 1990].

The use of sensitive and specific methods for the early diagnosis of HCMV infection is often required in monitoring transplant patients. It is essential for a timely application of antiviral therapy to prevent the life-threatening consequences of viral disease and also to detect the efficacy of the treatment. For this purpose, the demonstration of viral antigens in peripheral blood leukocytes (PMNLs) and of viral nucleic acids in the

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same cells or in sera has proven to be of valid support [The et al., 1992; Boland et al., 1992; Cunningham et al., 1995].

A specific and rapid assay for diagnosis and monitoring of an active HCMV infection was based on the detection of the lower matrix protein pp65 in peripheral blood leukocytes [Grefte et al., 1991; Ghisetti et al., 1996]. The number of positive cells (quantitative antigenaemia) has been demonstrated to well correlate with active infection [Gerna et al., 1992].

Polymerase chain reaction (PCR) on DNA extracted from circulating leukocytes has been used widely because of its sensitivity and specificity in detecting HCMV-DNA [Zipeto et al., 1992; Drouet et al., 1993; Lorino et al., 1993]. However, this method has a limited capacity when used to distinguish between replicating and latent virus [Kanj et al., 1996; Weber et al., 1994; Del Gado et al., 1992]. Some investigators, assuming that active viral replication may lead to the release of virus from cells into serum, performed HCMV-DNA PCR on plasma samples. It was shown that the positivity of plasma PCR preceded the detection in shell vial culture. Thus, the test has been considered suitable for screening patients at risk of HCMV infection [Nolte et al., 1995; Hebart et al., 1996].

Another useful approach in molecular biological methods to determine the presence of HCMV infection is evidence of specific HCMV mRNA. Reverse transcriptase-PCR (RT-PCR) is shown to be more indicative since the presence of HCMV transcripts in PMNLs is believed to correlate with an active viral infection [Meyer et al., 1994]. Furthermore RT-PCR has been developed for the detection of both immediate early, and late transcripts of HCMV in peripheral blood leukocytes [Meyer-Konig et al., 1995].

In this study a comparative evaluation of pp65 antigenaemia, PCR for HCMV-DNA, and Reverse Transcriptase (RT-PCR) for HCMV late mRNA was carried out in the follow-up of solid organ transplant patients, in order to define the best diagnostic protocol when monitoring high risk patients.

## PATIENTS AND METHODS

### Patients

Fifty patients, who received solid organ transplants at the Transplantation Unit of Policlinico Umberto I in Rome, were studied. Twenty nine underwent kidney transplant, 17 liver, and 4 of the lung. After the transplants, all patients received prophylactic therapy against HCMV infection. For kidney and liver transplants, patients were treated with oral administration of acyclovir for 1 month (400 mg/die 3 times daily); for lung transplants, intravenous ganciclovir was used for 1 month (10 mg/Kg/die). Patients received quadruple immunosuppressive therapy with cyclosporin A, azathioprine, methilprednisolone and anti-lymphocyte globulin. The dosage of immunosuppression and the treatment of rejection episodes were established for each patient group according to specific protocols that took into consideration patient clinical conditions. Patients symptomatic for HCMV infection were treated with ganciclovir at the dosage of 10 mg/Kg/die intravenously for 2 weeks. In 3 cases, specific anti-HCMV hyperimmune globulins were associated.

### HCMV Monitoring

After transplantation, patients were followed-up for HCMV infection for six months, according to the following protocol: in the first month pp65 antigens, PCR and HCMV antibodies were carried out weekly; in the second and third month samples from the patients were collected every 2 weeks. From the third to the sixth month patients were monitored once a month. In the case of a positive antigenaemia and/or PCR, RT-PCR for HCMV late mRNA was performed. Patients with evidence of clinical signs of HCMV infection were strictly monitored throughout the whole period.

### Clinical Definitions

A subject was considered to be infected with HCMV when pp65 antigen and/or HCMV DNA in PMNLs were detected. Moreover, HCMV seroconversion or specific IgM detection were considered to be a further sign of infection.

HCMV infection was defined as asymptomatic when no clinical symptoms or signs of laboratory abnormalities were detected. HCMV infection was considered symptomatic when active infection, revealed by antigenaemia and/or PCR, occurred in association with one or more of the following symptoms: fever, leukopenia, thrombocytopenia and/or evidence of organ invasion.

### PMNLs Sample Preparation

Blood specimens were processed within 2 hrs of collection; 1 ml of 6% dextran solution, molecular weight 70.000, was added to 5 ml of EDTA treated blood to obtain the PMNLs fraction. Cells were washed twice with saline buffer and diluted at $5 \times 10^6$ cells/ml.

### pp65 Antigen Test

Aliquots of $5 \times 10^4$ PMNLs were prepared on glass slides. Cells were fixed and permeabilised according to the manufacturer's instructions (CMV Ag-Kit, Argene). Then, cells were stained with a pool of anti-HCMV pk65 monoclonal antibodies and with fluorescein-conjugated anti mouse IgG + IgM $F(ab)_2$ fragment. Slides were examined under a fluorescent microscope (Orthoplan, Leitz Wetzlair, Germany), positive PMNLs were counted and referred to $2 \times 10^5$ cells.

### Polymerase Chain Reaction for HCMV DNA

PMNLs samples were stored at −70°C until DNA extraction. Five hundred μl of $5 \times 10^6$/ml PMNLs had undergone alkaline thermolysis and were processed according to the manufacturer's instruction (Bioline Diagnostics, s.r.l.). After extraction procedure DNA was resuspended in 100 μl of specific buffer corresponding to an average DNA concentration of 0.5 μg/ul. Ten ul of DNA extract, corresponding to $2.5 \times 10^5$ cells, was incubated with 90 ul of amplification mixture: KCl 50

TABLE I. PCR Primers and Specific Probe Relative to HCMV B-Glycoprotein Gene Sequence

| | |
|---|---|
| PCR up stream | $P2^{5'}CGAACATGGAATCCAGGATCTG^{3'}$ |
| PCR down stream | $P4^{5'}GTAGTGTTGTAGATAGTCTC^{3'}$ |
| PROBE | $PP^{5'}CGTCTTCTGAAGCCGTCAGTCATAG^{3'}$ |

mM, Tris HCl 10 mM, $MgCl_2$ 1.5 mM, gelatin 0.01%, deoxynucleotides triphosphate (dATP, dCTP, dGTP, dTTP) 200 uM each, 2.5 U Taq Polymerase (Perkin Elmer, Cetus) and 50 pMol of up and downstream primers (Table I). These primers amplified a 229 bp sequence of the HCMV B-glycoprotein gene. The PCR reaction was performed in an automated thermal cycler (DNA Thermal Cycler, Eppendorf) for 35 cycles (denaturation 94°C for 1 min; annealing 58°C for 1 min and 30 s, extension 72°C for 1 min; plus 10 min of extension in the last cycle). Twenty ul of each amplified product was analysed by 2% agarose gel electrophoresis, stained with ethidium bromide (0.5 ug/ml), and photographed over UV-light.

Amplified products were transferred from gels to nylon membrane (Hybond N+, Amersham International, UK) by the Southern technique [Tijssen, 1993] and hybridized with an internal probe (Table I) of 25 bp digoxigenine labelled at 3′ end. Hybrids were detected by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenine alkaline phosphatase conjugate) visualised by color reaction with 5′-bromo-4chloro-3indolil Phosphate (X-Phosphate) and Nitroblue Tetrazolium salt (NBT) (Boheringer Mannheim).

As a positive control in extraction and amplification steps, Peu cell line infected with AD169 and/or Towne strains was used. The same uninfected cell line was included as a negative control in each determination.

The sensitivity of the PCR technique for the detection of HCMV DNA after 35 amplification cycles and hybridization step corresponded to 10 viral genomes.

### Reverse-Transcriptase PCR (RT-PCR)

RT-PCR was carried out on RNA extracted from PMNLs. Fifty ul of $5 \times 10^6$ PMNLs were lysed with Guanidium Thiocyanate solution as described by Chomczinsky [Chomczynski et al., 1987].

Two ul of the total RNA extracted was transcribed to cDNA with 50 U of MuLv Reverse Transcriptase (Perkin Elmer, Cetus). cDNA generated by RT was amplified using a couple of primers relative to a 263 bp sequence of a late HCMV gene, encoding for the Major Capsid Protein (MCP) of nucleocapsid [Gozlan et al., 1992; Gozlan et al., 1993).

To check the effectiveness of RNA isolation, each sample was also tested for mRNA relative to cellular beta 2-globin gene.

Further controls were carried out to differentiate viral genomic DNA from cDNA amplified products. Since no introns are included in the HCMV MCP gene, each positive RNA sample was tested again by RT after DNAse digestion. For the same purpose, RNA extracted was directly amplified by PCR excluding the RT step. Amplified products were run in 2% agarose gel electrophoresis, then transferred to nylon membrane (Hybond N, Amersham International, UK) and hybridized with a 39 bp 3′ Dig-labelled probe.

### HCMV Serology

During the follow-up IgG and IgM to HCMV were measured by enzyme-linked immunoadsorbent assay (ELISA, Abbott). Both positive and equivocal specimens on initial testing in the IMX CMV IgM assay were tested again after treatment with Rheumatoid Factor Neutralisation Reagent.

### Statistical Analysis

Data of antigenaemia test were expressed and evaluated by Student's t Test. $P$ values $<0.05$ were considered significant.

## RESULTS

No clinical signs and/or laboratory evidence of HCMV infection were observed in 17 of 50 (34%) of transplant recipients and 33 (66%) had evidence of HCMV infection. Among the infected patients, 18 received kidney transplant, 13 the liver and 2 the lung (Table II). Results were analysed for the first HCMV symptomatic or asymptomatic infection episode from the time of transplant. The first sign of infection, such as positive antigenaemia and/or viral DNA, occurred between the $2^{nd}$ and the $10^{th}$ week after transplantation, except for one patient in which they appeared at the $20^{th}$ week. Sixteen of the infected patients (48.5%) including 12 kidney and 4 liver transplant recipients, developed HCMV asymptomatic infection between the $2^{nd}$ and the $10^{th}$ week. They did not undergo antiviral treatment (Fig. 1). The other 17 patients (51.5%), 9 liver, 6 kidney and 2 lung transplant recipients, showed clinical signs of HCMV infection between the $3^{rd}$ and the $8^{th}$ week except for two in the $10^{th}$ and $12^{th}$ week. (Fig. 2). The most frequent symptoms were fever observed in all symptomatic patients, leukopenia in 9 and increasing creatinemia in 8 out of 17. Three patients developed interstitial pneumonia.

### Antigenaemia

pp65 antigen was found in 5 asymptomatic patients and in all the symptomatics. The appearance of HCMV antigen in asymptomatic patients ranged between the $3^{rd}$ and the $10^{th}$ week after the transplants and the persistence of positivity was for 3–8 weeks. In 3 asymptomatic patients, antigenaemia became positive at the same time of PCR while in the other two patients pp65 antigen appeared respectively 1 and 2 weeks after viral DNA. As shown in Figure 1, the antigenic load relative

TABLE II. Six Months HCMV Follow-Up in 50 Solid Organ Recipients

| | Kidney | Liver | Lung |
|---|---|---|---|
| Symptomatic | 6 | 9 | 2 |
| Asymptomatic | 12 | 4 | — |
| No Infection | 11 | 4 | 2 |

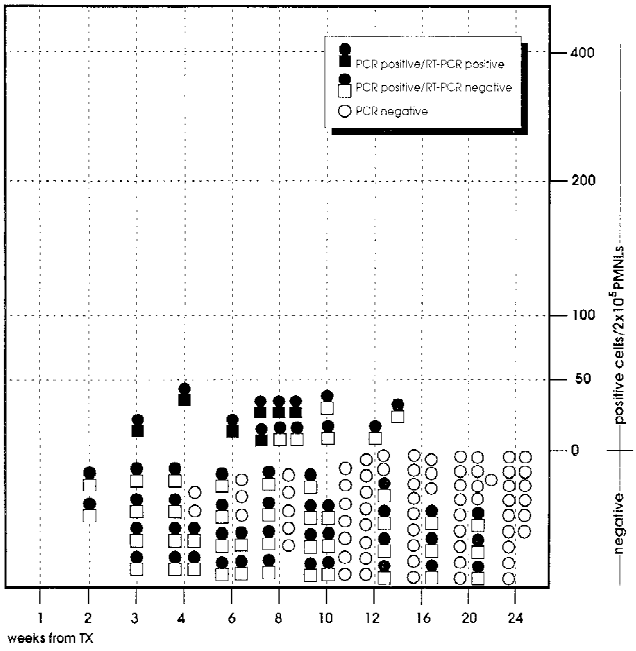


Fig. 1. Monitoring of transplant recipients for HCMV infection by pp65 antigenaemia, PCR and RT-PCR. Data are referred to **asymptomatic** patients from the first evidence of infection.

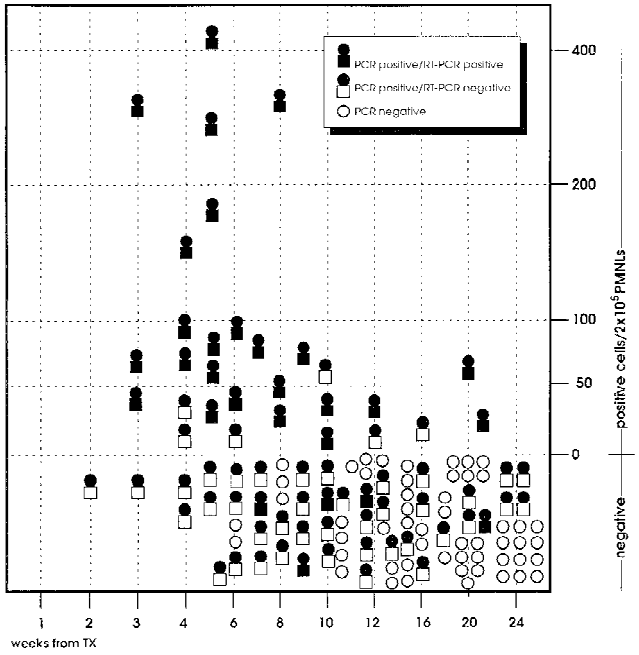


Fig. 2. Monitoring of transplant recipients for HCMV infection by pp65 antigenaemia, PCR and RT-PCR. Data are referred to **symptomatic** patients from the first evidence of infection.

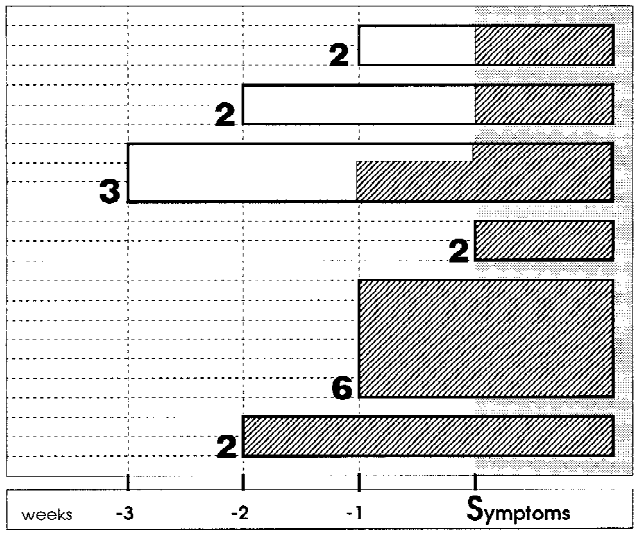


Fig. 3. Detection of pp65 and HCMV-DNA related to the onset of clinical symptoms. The open bars show PCR positivity; antigenaemia plus DNA positivity are expressed by hatched bars. Bars' width refers to the number of patients.

to the asymptomatic cases ranged between an average value of 31 ± 11 positive cells/2 × $10^5$ $PMNL_s$.

In the symptomatic patients pp65 antigen was detected between the 3rd and the 8th week after the transplant; in these patients positive values persisted until the 2nd and the 7th week from the start of ganciclovir therapy. In 7 symptomatic patients antigenaemia became positive after 1–3 weeks from DNA detection. In the other 10 patients who developed clinical disease, DNA and pp65 antigen were simultaneously revealed. In 2 symptomatic patients, pp65 antigen resulted positive two weeks before the onset of clinical symptoms, in 8 one week before and in 7 at the onset of HCMV disease (Fig. 3). The antigenic load was elevated more significantly in symptomatic patients than in asymptomatics ($P < 0.01$) and it was correlated to the clinical status of the patients: antigenic levels > 100/2 × $10^5$ $PMNL_s$ were associated to fever and leukopenia. The highest levels were observed in 3 patients in coincidence of organ involvement. In these cases the number of positive cells was >250/2 × $10^5$ $PMNL_s$ (Fig. 2).

## HCMV DNA

PCR detected HCMV DNA relative to a 229 bp sequence of B-glycoprotein gene in all symptomatic and asymptomatic patients. The positivity was detectable between the 2nd and 10th week except for 1 symptomatic patient in which HCMV DNA appeared in the 20th week. In 15 patients who developed HCMV disease, viral DNA was detected 1–3 weeks before the onset of clinical symptoms (Fig. 3). From the first evidence, HCMV DNA persisted for 2–10 weeks. Each positive amplified product was confirmed by Southern blotting and hybridization by the specific probe.

TABLE III. Outcome of Antigenaemia, PCR for HCMV-DNA and RT-PCR for Late HCMV mRNA in Symptomatic Patients

| | Weeks after the start of therapy[b] | DNA | late mRNA | pp 65-antigen[a] |
|---|---|---|---|---|
| Group 1[c] | 1 | + | + | 136.8 ± 115.7 |
| (5 pts) | 2–3 | + | − | 85.6 ± 41.4 |
| | 4 | − | − | − |
| Group 2 | 1–2 | + | + | 252.0 ± 183.6 |
| (3 pts) | 3–5 | + | − | − |
| | 6 | + | + | − |
| | 7 | + | + | 44.0 ± 26.2 |
| | 8 | − | − | − |
| Group 3 | 1–2 | + | + | 105.6 ± 142.9 |
| (5 pts) | 3–5 | + | − | − |
| | 6 | − | − | − |
| Group 4 | 1–2 | + | + | 62.6 ± 22.0 |
| (3 pts) | 3–end of follow-up | + | − | − |
| Group 5[d] | 1 | + | + | 44 |
| (1 pts) | 2–3 | + | + | 250 |

− negative; + positive.
[a]N of pp 65 positive cells/2 × $10^5$PMNLs.
[b]The start of therapy corresponded to the appearance of HCMV clinical symptoms.
[c]Groups represent patients with similar HCMV infection outcome.
[d]Patient died of HCMV pneumonia.

### RT-PCR

The study of HCMV late transcripts showed that in 5 cases of asymptomatic infection, HCMV was replicating in host cells. In these patients low levels of pp65 antigen were detected. Moreover RT-PCR was positive in all symptomatic patients in coincidence with clinical signs of acute HCMV disease. In 10 symptomatic patients late mRNA of HCMV was detected at the same time as the DNA, while in 7 cases it appeared one week later.

During the follow-up of patients treated with ganciclovir (Table III), RT-PCR in 5 patients resulted negative after the first week of therapy, while antigenaemia and PCR were still positive for another week. In 3 other patients, 2 liver recipients and 1 of kidney, both RT-PCR and antigenaemia were negative at the end of ganciclovir therapy while PCR remained positive 3 weeks further. After this period, the reappearance of late mRNA was observed before pp65 antigen detection and clinical relapse. The ensuing new cycle of ganciclovir associated with anticytomegalovirus hyperimmune globulin allowed a complete recovery. In other 8 symptomatic patients, RT-PCR and antigenaemia were negative at the end of ganciclovir administration while HCMV DNA was still detectable for three weeks in 5 of them and for the whole period of observation in the others. In none of these patients clinical relapse of HCMV illness was observed. One symptomatic patient showed the persistence of positivity to all tests from the 3rd week after transplantation and, despite the antiviral treatment, he died of HCMV pneumonia.

Serological monitoring showed IgM increase in all asymptomatic patients except for one in which the IgM value was continuously lower than the cut-off. IgM were positive in 12 of the symptomatic patients simultaneously with the HCMV infection. IgM mean values did not reveal any significant differences between asymptomatic and symptomatic patients. In the latter group a weak correlation between the low IgM values and the persistence of antigenaemia and/or HCMV-DNA was observed (data not shown).

## DISCUSSION

Because of the serious consequences of HCMV infection in transplant recipients, rapid and specific methods of diagnosis are required. The best diagnostic procedure should provide an accurate way to establish an early diagnosis in the course of infection or disease leading to specific antiviral therapy [Kanj et al., 1996].

Recently, an HCMV pp65 antigen test in PMNLs has been demonstrated as a sensitive and specific method to detect the HCMV viremia. It has been reported that this test allows a more careful clinical evaluation of HCMV infection than the viral isolation from clinical specimens [Landry et al., 1993]. The high number of leukocytes expressing pp65 antigen well correlate with the disease while a low number is usually associated with asymptomatic infection [Gerna et al., 1991; Gomez et al., 1992].

The rapidity of diagnosis and monitoring of HCMV infection have been further improved by the detection of viral nucleic acid in blood, carried out by amplification methods. The lack of a predictive value is the major draw back of PCR test. To overcome this limitation, some authors have reported quantitative or semiquantitative methods to monitor the viral load and antiviral therapy [Drouet et al., 1995; Fox et al., 1995; Rawal et al., 1994]. Nonetheless, the interpretation of the results needs to be standardised with reference to a cut-off value to exclude clinically insignificant low-level results, especially in the case of latent HCMV infection [Fernando et al., 1994].

At present, the application of the RT-PCR method has been suggested for the study of the HCMV transcripts for IE and late proteins in blood peripheral cells as a measure of active viral replication [Bitsch et al., 1993; Randhawa et al., 1994; Velzing et al., 1994]. A matter of greater interest is in targeting transcripts of late viral protein genes [Nelson et al., 1996], since these may be more indicative of active viral replication. Such gene transcription can discriminate latent from replicating HCMV more accurately than IE mRNA detection, since IE genes are also transcribed during nonproductive infection [Taylor-Wiedeman et al., 1994; Einhorn et al., 1984].

The study was designed to establish a diagnostic protocol which may allow a better follow-up of the HCMV infection. For this purpose, the results obtained by antigenaemia, PCR and late HCMV mRNA were analysed. We chose to perform PCR on DNA extracted from leukocytes because the positive specimens were also suitable for mRNA amplification.

As reported by some investigators [Kanj et al., 1996; Nelson et al., 1996] the positivity of PCR does not discriminate HCMV productive from latent infection; in fact in our study viral DNA was detected indepen-

dently from the onset of any clinical sign. Nevertheless, in 7 symptomatic and in 2 asymptomatic patients, PCR resulted positive before antigenaemia.

The antigenaemia test correlated well with the evolution of HCMV infection; it resulted positive in all symptomatics and in 31.2% of asymptomatics, but the antigenic load was significantly lower in the second group, which never exceeded $42/2 \times 10^5$ PMNLs. In the symptomatics the high number of pp65 positive cells ($>100/2 \times 10^5$) was always associated with clinical signs of illness; this allowed, in short time, to make diagnosis and to begin the antiviral therapy.

A moderate level of antigenaemia remained detectable in 5 out of 17 patients that underwent ganciclovir therapy, even after the disappearance of symptoms. In these patients late transcript detection was more significant, indicating cessation of viral replication due to antiviral therapy prior to antigenaemia. On the contrary in the case of the patient that died of HCMV pneumonia, despite the antiviral therapy, late mRNA never disappeared completely maybe reflecting the resistance of the virus to ganciclovir. Moreover, in the treatment of 3 patients RT-PCR for late transcripts became positive after the end of ganciclovir administration and before antigenaemia and clinical symptoms recurred. This indicated a viral relapse, thus allowing the start of a new cycle of antiviral therapy.

The data suggest that RT-PCR was the procedure that showed the trend of HCMV infection in symptomatic patients more accurately even though the antigenaemia was very useful in monitoring the patients after the allograft. The discrepancy between late m-RNA and antigenaemia assay may be explained by the results described by Grefte et al. [1994]. They demonstrated that the expression of pp65 in peripheral blood cells, without corresponding mRNA, indicates that the presence of this protein is the result of uptake by phagocytic leukocytes rather than de novo synthesis [Grefte et al., 1994]. These data suggest that during HCMV infection the evaluation of late mRNA and pp65 antigen in PMNLs may show the expression of two different biological events related to the replicative viral cycle during the human infection.

A major consideration in the use of RT-PCR for the diagnosis of the transcriptional activity of a DNA virus is to distinguish between the cDNA derived from the reverse transcription step and the genomic DNA of the virus itself. This becomes extremely important in the presence of unspliced transcripts. We performed RT-PCR on the basis of the Gozlan report [Gozlan et al., 1993]. The primers used were related to a region in which no evidence of spliced transcripts was found. In our study, we tested controls to exclude genomic DNA contamination after total cellular RNA extraction, and to be sure that the amplification product was relative only to messenger RNA. In a recent report, Nelson et al. [1996] developed a RT-PCR that detects the spliced transcript of an HCMV late gene (R270805) which yields a DNA product smaller than the amplification product of viral genomic DNA. This could improve and simplify the standardization of RT-PCR for late transcripts of HCMV.

In conclusion, our findings suggest that pp65 antigen is a reliable marker of HCMV infection, useful in the monitoring of transplant recipients for its simple application. Late transcript detection seems to be more indicative in the follow-up of symptomatic patients during the antiviral therapy giving timely information on virus replicative status. This approach needs better standardisation for enhancement of the diagnostic aspect for selecting and monitoring patients at high risk of HCMV disease.

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